In Vitro Assay for Trans-Phosphorylation of Rhodopsin by Rhodopsin Kinase†

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ABSTRACT: Trans-phosphorylation of rhodopsin refers to a reaction in which a rhodopsin kinase molecule that has been activated by a light-activated rhodopsin molecule collides with and phosphorylates a second molecule of rhodopsin that has not been activated by light. It has been invoked as a mechanism for high-gain phosphorylation, a phenomenon that is observed at low bleaching levels where up to several hundred moles of phosphate are added to the rhodopsin pool per mole of photolyzed rhodopsin. Trans-phosphorylation is an appealing mechanism to propose for high-gain phosphorylation, but it has not been tested directly because of the difficulty inherent in unambiguous identification of light-activated and dark forms of rhodopsin present in the same reaction mixture. We report here a direct assay for trans-phosphorylation of rhodopsin. The assay is based on the use of a split receptor mutant of rhodopsin, SR(1−4/5−7), in which the fully functional protein is assembled from two separately expressed fragments. Because of different electrophoretic mobilities, SR(1−4/5−7) and wild-type rhodopsin can be monitored independently for phosphorylation while in the same reaction mixture. Thus, if wild-type rhodopsin is exposed to light and then incubated in the dark with SR(1−4/5−7), ATP, and rhodopsin kinase, phosphorylation of SR(1−4/5−7) would be a clear demonstration that trans-phosphorylation has occurred. Despite numerous attempts using several different experimental configurations, we have been unable to detect trans-phosphorylation of dark rhodopsin with this system.

Rhodopsin kinase belongs to the GRK family of serine/threonine protein kinases that specifically recognize the activated forms of G protein-coupled receptors (Palczewski & Benovic, 1991; Lorenz et al., 1991; Inglese et al., 1993). Phosphorylation of light-activated rhodopsin leads to the binding of arrestin in a reaction that competes with further activation of the G protein transducin and helps to bring about termination of the photoresponse (Wilden et al., 1986; Bennett & Sitaramayya, 1988).

The mechanism of the phosphorylation reaction, particularly the mechanism of regulation by light, has been the subject of numerous studies (Buczylko et al., 1991; Dean & Akhtar, 1993, 1996; Kühn, 1984; Palczewski et al., 1993; Pulvermuller et al., 1993). Until recently, the activity of the kinase was thought not to be regulated, and the effect of light on the reaction was presumed to be restricted to conformational transitions in rhodopsin (Kühn, 1984). Accordingly, the phosphorylation sites on rhodopsin were thought to be inaccessible in the dark and to become exposed for phosphorylation by the kinase only after photoconversion of rhodopsin to the active metarhodopsin II (MII; Matthews et al., 1963; Emeis et al., 1982) conformation. However, the results of studies with synthetic peptide substrates for the kinase appear to be at odds with this simple model. In particular, Fowles et al. (1988) and Palczewski et al. (1991) showed that a peptide derived from the carboxy terminus of rhodopsin and containing all known phosphorylation sites could serve as a substrate for the kinase, but only if rhodopsin was also present in the reaction mixture and if the proteins were exposed to light. These data suggest that rhodopsin kinase resides normally in a dormant conformation and becomes active only after interaction with light-activated MII. Once activated, the kinase then phosphorylates even poor substrates such as the synthetic peptides.

Since the first observations of the light-dependent phosphorylation of rhodopsin a quarter century ago (Kühn & Dreyer, 1972; Bownds et al., 1972), it has been known that at low bleaching levels (e.g., <1% rhodopsin bleached) up to several hundred moles of phosphate are added to the rhodopsin pool per mole of photolyzed rhodopsin. Given that there are only 348 amino acids in rhodopsin and of these only nine have been shown to be phosphorylated (Wilden & Kühn, 1982), a rather straightforward conclusion from these results is that non-photolyzed rhodopsin is phosphorylated in a light-dependent manner in this reaction. This phenomenon has been referred to as high-gain phosphorylation (Binder et al., 1990). High-gain phosphorylation has been observed in living frogs (Binder et al., 1996), in electropermeabilized frog photoreceptor outer segments (Binder et al., 1990), and in urea-stripped bovine photoreceptor outer segments reconstituted with purified rhodopsin kinase (Chen et al., 1995). It has been invoked as a possible mechanism for light adaptation, although more recent studies suggest that the quantitative aspects of the reaction are insufficient to account for adaptive responses *in vivo* (Binder et al., 1996).

The model for light-dependent activation of rhodopsin kinase described above provides a satisfying rationale for

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the mechanism of high-gain phosphorylation. At low bleaching levels, non-photolyzed or "dark rhodopsin" is in large molar excess over light-activated or MII-forms. Therefore, any rhodopsin kinase molecule that has been activated by MII is far more likely to encounter a dark rhodopsin by diffusional collision (either free from the activating rhodopsin or as a binary complex in the lipid bilayer) than a second MII. Once the kinase has been activated, these secondary collisions result in efficient phosphorylation of the dark rhodopsins despite the fact that the rhodopsin molecules have not themselves been activated by light. An implicit assumption in this mechanism is that light-dependent conformational changes do not control exposure of the phosphorylation sites in rhodopsin and that the sites are accessible to the activated kinase in both the dark and MII states.

Despite the appeal of this mechanism, it must be embraced with some caution because the model for phosphorylation of dark rhodopsin by active rhodopsin kinase (a reaction that we will refer to as "trans-phosphorylation") has never been directly tested. Prior to this work it has not been possible to unambiguously distinguish light-activated and dark forms of rhodopsin recovered from the same phosphorylation reaction mixtures. We present here an assay for trans-phosphorylation of rhodopsin. This assay was made possible by the use of a split rhodopsin mutant, SR(1−4/5−7), in which the protein is assembled in functional form from two separately expressed fragments, one containing transmembrane segments 1−4 and the other containing transmembrane segments 5−7 (Yu et al., 1995). Since all of the phosphorylation sites are located along the carboxy terminus of the protein (Hargrave & McDowell, 1992), phosphorylation of SR(1−4/5−7) results in modification of the smaller fragment containing transmembrane segments 5−7. Therefore, the use of SR(1−4/5−7) allows a biochemical distinction to be established for phosphorylated forms of full-length and split rhodopsin on the basis of differential electrophoretic mobility on SDS−PAGE gels. If, for example, the wild-type rhodopsin is exposed to light and then mixed in the dark with rhodopsin kinase, [γ-^{32}P]ATP, and SR(1−4/5−7), phosphorylation of dark SR(1−4/5−7) can be monitored without interference from phosphorylation of the wild-type protein because the two signals appear in different locations on the autoradiogram. Thus, trans-phosphorylation of dark SR(1−4/5−7) can be monitored unequivocally in the presence of light-activated molecules of wild-type rhodopsin. In some experiments, we wish to reconstitute both proteins into the same asolectin vesicles. This arrangement precludes selective activation of either protein by light, so we use a constitutively active mutant, K296G, in place of the wild-type protein so that the kinase can be activated without illumination of the reaction mixture (Rim & Oprian, 1995).

After numerous attempts and several different experimental arrangements, we have been unable to detect phosphorylation of dark rhodopsin in this system.

EXPERIMENTAL PROCEDURES

Materials. Unless noted otherwise in the text, all materials including the 1D4-Sepharose 4B anti-rhodopsin immunoaffinity matrix and peptide I eluant (peptide 1 contains the epitope for 1D4) were as described by and from the same sources as reported in Rim and Oprian (1995).

Opsin Mutants. The wild-type opsin gene used in these studies was a synthetic gene encoding the amino acid sequence of native bovine rhodopsin (Ferretti et al., 1986). Mutations were constructed by cassette mutagenesis, and all mutant opsins used in this study have been described in previous publications: K296G, [see Zhukovsky et al. (1991), Robinson et al. (1992), and Rim and Oprian (1995)]; SR-(1−4/5−7) [see Yu et al. (1995)]. These references should be consulted for details on construction of the mutant genes and the initial characterization of the proteins isolated from transfected COS cells.

Expression of Wild-Type and Mutant Opsin Genes in COS Cells. The opsin genes were expressed transiently in transfected COS cells according to previously described protocols (Oprian et al., 1987; Oprian, 1993). For the experiment of Figure 6 in which K296G and SR(1−4/5−7) were co-expressed in the same COS cells, 2 μg of each plasmid (per 100-mm culture dish) were combined and used to transfect the cells, as has been described previously for co-expression of gene fragments for SR(1−4/5−7) and other split rhodopsin mutants (Yu et al., 1995).

Purification of Wild-Type and Mutant Opsins from Transfected COS Cells. With the exception of SR(1−4/5−7), the wild-type and mutant opsins were purified from transfected COS cells in their apoprotein or opsin forms and then combined with the 11-*cis*-retinal chromophore after reconstitution of the proteins into asolectin vesicles (Rim & Oprian, 1995). In contrast, SR(1−4/5−7) was combined with 11-*cis*-retinal immediately before solubilization of the harvested cells with CHAPS (Yu et al., 1995) and then purified and reconstituted into vesicles exactly as described below except that all procedures were carried out in the dark with illumination from a 15 watt incandescent bulb filtered through a Kodak Safelight #2 filter.

Transfected COS cells from 10 100-mm culture plates were harvested by scraping with a rubber policeman in 1 mL/plate 10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl (PBS). The suspended cells were combined into a 15-mL conical, polypropylene culture tube and collected by centrifugation for 3 min in a clinical centrifuge. The supernatant fraction was discarded and the cells were washed by resuspending the pellet in 10-mL of 50 mM Tris buffer, pH 7.0, containing 140 mM NaCl and 1 mM dithiothreitol. The cells were collected again by centrifugation in the clinical centrifuge, the supernate was discarded, and the cell pellet was resuspended in 50 mM Tris buffer, pH 7.0, containing 140 mM NaCl, 1 mM dithiothreitol, 1% (w/v) CHAPS, and 1 mg/mL asolectin. This suspension was incubated with mixing at 4 °C for 20 min to solubilize cellular membranes and then fractionated by centrifugation for 3 min in a clinical centrifuge. The nuclear pellet was discarded and the supernate transferred to a fresh 15-mL conical, polypropylene tube. 1D4-Sepharose 4B matrix (150 μL of packed gel) was added and the suspension incubated at 4 °C with mixing for 45 min. The immunoaffinity beads were then collected by centrifugation for 1 min in a clinical centrifuge, the supernate was discarded, and the beads were transferred to a 1-mL polypropylene syringe barrel containing a glass wool plug in the bottom. The syringe was then placed in a 15-mL conical, polypropylene tube, and the beads washed by centrifugation 10 times with 1 mL of solubilization buffer. The protein was eluted from the solid support by resuspending the beads in 300 μL of solubilization buffer containing

50 μM peptide I followed by a 20 min incubation on ice. The eluant was collected by centrifugation, the elution procedure was repeated with a second 300 μL aliquot of peptide I, and the two fractions were combined for further use.

Reconstitution of Opsins into Lipid Vesicles. The eluant (approximately 600 μL) from the 1D4-Sepharose 4B matrix was applied to a 1 × 10 cm column of Sephadex G-50 that had been equilibrated with 10 mM Tris buffer, pH 7.4, containing 1 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. The gel filtration column was developed under gravity flow with the same buffer, and 500 μL fractions were collected and analyzed for light-scattering by recording the absorption spectrum from 650 to 500 nm. Turbid fractions from the void volume (usually two to three fractions) were pooled, and the vesicles were concentrated approximately 10-fold by centrifugation in a Centricon-30 concentrator (Amicon) for 30 min at 7000 rpm in a Beckman JA-20 rotor. The concentrated vesicles were collected (total volume approximately 150 μL) and stored at 4 °C until use.

Co-reconstitution of K296G and SR(1−4/5−7) into asolectin vesicles (Figure 6) was performed exactly as described above. We estimate that the concentration of each protein was at least 10-fold greater than that of the vesicles, making it likely that some vesicles contain both K296G and SR(1−4/5−7). The asolectin vesicle concentration was estimated to be 0.15 μM from (1) the average molecular weight of asolectin lipids (1000 g/mol); (2) the average radius (17 nm) of similar vesicles determined by electron microscopy (Sarti et al., 1995); and (3) the average lipid surface area (39 Å) for a lipid head-group (Gennis, 1989). Fusion of vesicles was accomplished by freezing the sample overnight at −20 °C and then thawing at room temperature before use. This procedure increases vesicle size by 10-fold over that of untreated samples (Kasahara & Hinkle, 1977).

Urea-Stripped Photoreceptor Outer Segments. Procedures for preparation of urea-stripped bovine photoreceptor outer segments were performed according to a previously described protocol (Rim & Oprian, 1995).

Purification of Rhodopsin Kinase from Sf9 Cells. Recombinant rhodopsin kinase was purified from Sf9 cells by chromatography on a recoverin affinity column exactly as has been described by Chen et al. (1995). The specific activity of the purified protein was determined to be 240 nmol of phosphate/min/(mg of rhodopsin kinase) by phosphorylation of urea-stripped bovine photoreceptor outer segments with [γ^{32}P]ATP (1000 cpm/pmol) as described by Palczewski (1993). Protein concentration was determined by the method of Bradford (1976).

Assay for Phosphorylation by Rhodopsin Kinase. Phosphorylation of wild-type and mutant opsins was measured using the purified COS cell expressed proteins after reconstitution into asolectin vesicles and incubation with excess 11-*cis*-retinal to regenerate the holoproteins (except for K296G which does not bind 11-*cis*-retinal and, therefore, remains in the constitutively active state; Zhukovsky et al., 1991; Robinson et al., 1992; Rim & Oprian, 1995). The reaction mixture contained in a total volume of 30 μL 75 mM Tris buffer, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 100 μM [γ-^{32}P]ATP (2000 cpm/pmol), 1 nM purified rhodopsin kinase, and 15 pmol of rhodopsin (the concentration of opsin was estimated from Western blots). For light-dependent phosphorylations, the reactions were initiated by exposure to white light which was continuous throughout the incubation period. For dark incubations, the reactions were initiated by the addition of [γ-^{32}P]ATP. The reactions were allowed to proceed for 40 min, and then stopped by the addition of 8 μL of 5× SDS load buffer (300 mM Tris buffer, pH 6.8, 10% SDS, 30% sucrose, and 0.026% bromophenol blue) and 2 μL of β-mercaptoethanol. The proteins were then separated by SDS−PAGE (the entire sample was loaded onto 12% gels: 12% acrylamide, 0.32% bisacrylamide), the gels dried on filter paper using a Bio-Rad model 583 gel dryer (1 h at 60 °C), and the phosphorylated proteins detected by autoradiography on Kodak X-OMAT AR film using an intensifying screen at −70 °C.

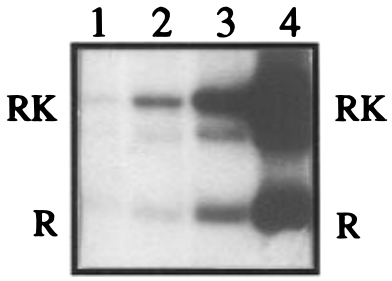

FIGURE 1: Dependence of rhodopsin phosphorylation in the dark on the concentration of rhodopsin kinase. The figure shows an autoradiogram from a reaction in which 500 nM rhodopsin (from bovine photoreceptor outer segments) was incubated with [γ-^{32}P]-ATP and increasing concentrations of rhodopsin kinase in the dark under conditions as detailed in Experimental Procedures. The reactions were allowed to proceed for 40 min. Rhodopsin kinase concentrations were as follows: lane 1, 10 nM; lane 2, 50 nM; lane 3, 100 nM; lane 4, 1 μM. R indicates location of rhodopsin on the autoradiogram; RK indicates the location of autophosphorylated rhodopsin kinase.

Final concentrations of 1 nM rhodopsin kinase and 500 nM rhodopsin were selected for routine trans-phosphorylation assays because higher concentrations of either protein resulted in a significant dark reaction in which rhodopsin was found to be phosphorylated by the kinase without exposure of the protein to light. As is shown in Figure 1, phosphorylation of a constant concentration of rhodopsin (500 nM) increases with increasing rhodopsin kinase concentration in the range 10 nM to 1 μM. This dark reaction was also observed if the rhodopsin kinase concentration was held constant at 1 nM and the rhodopsin concentration increased to 25 μM (not shown), as would be expected from the effects of mass action. Because of the possible influence of protein concentration on the trans-phosphorylation assay, we performed control experiments (Figure 8) to confirm that the results and conclusions from experiments at the lower concentrations applied also at higher concentrations.

RESULTS

Light-Dependent Phosphorylation of SR(1−4/5−7). The split rhodopsin mutant SR(1−4/5−7) used in this study was produced by co-expression of two separate gene fragments in COS cells, one fragment containing transmembrane segments 1−4 of rhodopsin and the other fragment containing transmembrane segments 5−7, as described previously (Yu et al., 1995). The protein is split between Pro-194 and His-195 in the second extracellular loop connecting transmembrane segments 4 and 5. As reported earlier (Yu et al., 1995), SR(1−4/5−7) expression level, ability to form a pigment with 11-*cis*-retinal, and specific activity for light-

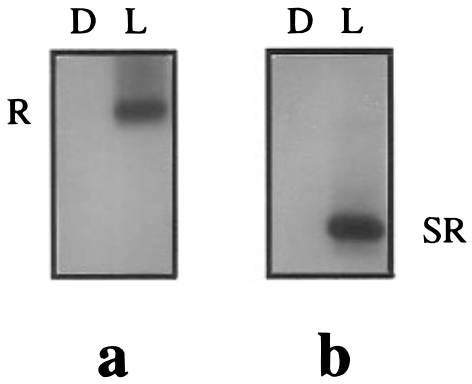

FIGURE 2: Light-dependent phosphorylation of the split rhodopsin mutant SR(1−4/5−7) by rhodopsin kinase. The figure shows autoradiograms of wild-type rhodopsin and the split rhodopsin mutant SR(1−4/5−7) after phosphorylation by rhodopsin kinase in the dark and after exposure to light. Both wild-type rhodopsin and SR(1−4/5−7) were purified from transfected COS cells. (a) Phosphorylation of wild-type rhodopsin. (b) Phosphorylation of the split rhodopsin mutant SR(1−4/5−7). Lane D, phosphorylation reaction carried out in the dark; lane L, phosphorylation after exposure to light. Assay conditions were as described in Experimental Procedures, and reactions were allowed to proceed for 40 min. R indicates location of wild-type (full-length) rhodopsin; SR indicates location of split rhodopsin mutant SR(1−4/5−7). Note that since all phosphorylation sites are on the carboxyl-terminus of rhodopsin, only the smaller fragment, containing transmembrane segments 5−7, is observed for SR(1−4/5−7).

dependent activation of transducin were comparable to wild-type rhodopsin. As is shown in Figure 2b, SR(1−4/5−7) was also phosphorylated by rhodopsin kinase in a light-dependent reaction that is similar to that observed with the wild-type protein (Figure 2a). Most importantly for the trans-phosphorylation assay, the phosphorylation sites are all located in the smaller fragment of SR(1−4/5−7), containing transmembrane segments 5−7, which is clearly resolvable from the full-length, wild-type protein by SDS−PAGE.

Assay for Trans-Phosphorylation of Rhodopsin. We have exploited the ability to resolve SR(1−4/5−7) from wild-type rhodopsin to develop an assay for trans-phosphorylation. First, the two proteins were separately reconstituted into asolectin vesicles. Wild-type rhodopsin was then exposed to light for 30 s and mixed in the dark with rhodopsin kinase, [γ-^{32}P]ATP, and the SR(1−4/5−7) sample which was not exposed to light. The mixture was allowed to react in the dark for 40 min after which it was assayed by SDS−PAGE and autoradiography for phosphorylation of SR(1−4/5−7). If a rhodopsin kinase molecule that had been activated by light-activated rhodopsin could phosphorylate a SR(1−4/5−7) molecule that had not been activated by light then we should have observed two labeled bands in the autoradiogram: one corresponding to the electrophoretic mobility of the full-length, wild-type rhodopsin and the other corresponding to the electrophoretic mobility of the smaller SR-(5−7) fragment. As is shown in Figure 3a, only one band, corresponding to the electrophoretic mobility expected for the light-activated wild-type rhodopsin, was observed for this reaction (lane D). As a control, the reaction was performed under continuous exposure to white light (lane L) and labeled bands were observed for both the wild-type rhodopsin and the SR(5−7) fragment, demonstrating that SR(1−4/5−7) was active and capable of being phosphorylated by the kinase under these conditions.

To determine if a higher concentration of ATP than the 100 μM used in Figure 3a would promote the trans-phosphorylation reaction we increased the ATP concentration to 3 mM (Dean & Akhtar, 1996). As is shown in Figure 3b, only the light-activated, wild-type rhodopsin was phosphorylated during incubation with rhodopsin kinase and 3 mM ATP in the dark. SR(1−4/5−7) was phosphorylated only after the reaction mixture was exposed to light.

Decreasing the ratio of light-activated/dark rhodopsin from 1/1 (Figure 3a) to 1/10 (Figure 3c) or 1/1000 (Figure 3d) also did not result in phosphorylation of the dark rhodopsin.

The reciprocal experiment was also performed as shown in the bottom panel of Figure 3. SR(1−4/5−7) was exposed

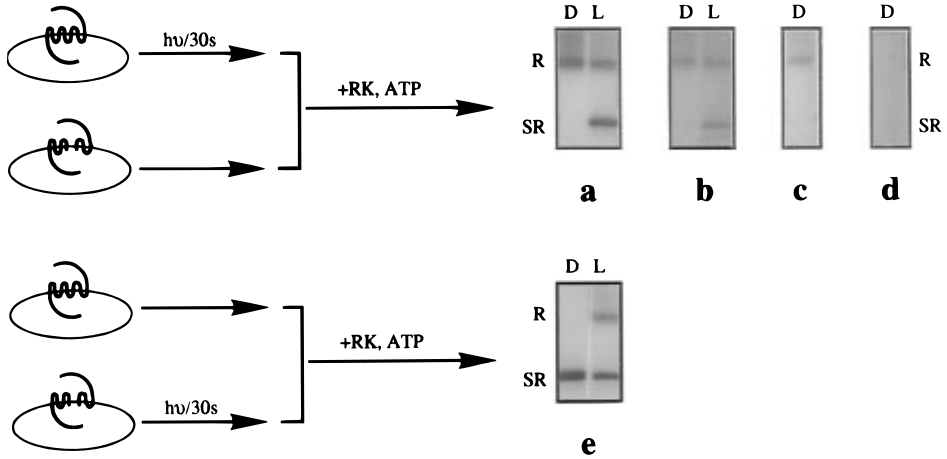

FIGURE 3: Assay for trans-phosphorylation of rhodopsin by rhodopsin kinase. (a−d) Vesicles containing wild-type rhodopsin were exposed to light for 30 s and then mixed and incubated with rhodopsin kinase, [γ-^{32}P]ATP, and vesicles containing the split rhodopsin mutant SR(1−4/5−7) either in the dark (lane D) or under continuous exposure to light (lane L). (e) Reciprocal experiment in which the split rhodopsin mutant SR(1−4/5−7) was exposed to light for 30 s and then mixed and incubated with rhodopsin kinase, [γ-^{32}P]ATP, and vesicles containing wild-type rhodopsin either in the dark (lane D) or under continuous exposure to light (lane L). Final concentrations were as follows: (a) Wild-type rhodopsin, 0.5 μM; SR(1−4/5−7), 0.5 μM; [γ-^{32}P]ATP, 100 μM. (b) Same as in a except that the [γ-^{32}P]-ATP concentration was 3 mM. (c) Same as in a except that the concentration of wild-type rhodopsin was 50 nM (i.e., 1/10 ratio of light-activated to dark rhodopsin). (d) Same as in a except that the concentration of wild-type rhodopsin was 0.5 nM (i.e., 1/1000 ratio of light-activated to dark rhodopsin). (e) Same as in a. Reaction conditions were as described in Figure 2 and Experimental Procedures. Both wild-type and split rhodopsins were purified from transfected COS cells. Logos in the figure are drawn to indicate vesicles containing either the full-length wild-type rhodopsin or the split mutant SR(1−4/5−7). *hυ*/30s indicates a 30 s exposure of the sample to light; R indicates location of the wild-type rhodopsin; SR indicates location of the split rhodopsin mutant SR(1−4/5−7).

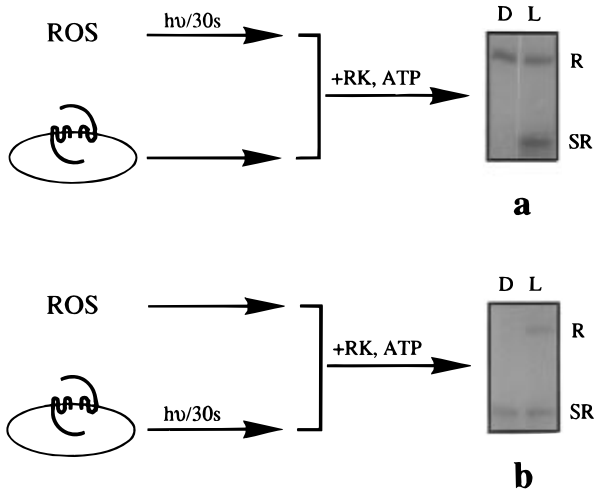

FIGURE 4: Assay for trans-phosphorylation of rhodopsin in bovine photoreceptor outer segments. (a) Bovine photoreceptor outer segments containing wild-type rhodopsin were exposed to light for 30 s and then mixed and incubated with rhodopsin kinase, [γ-^{32}P]-ATP, and vesicles containing the split rhodopsin mutant SR(1−4/5−7) either in the dark (lane D) or under continuous exposure to light (lane L). (b) Reciprocal experiment in which the split rhodopsin mutant SR(1−4/5−7) was exposed to light for 30 s and then mixed and incubated with rhodopsin kinase, [γ-^{32}P]ATP, and bovine photoreceptor outer segments containing wild-type rhodopsin either in the dark (lane D) or under continuous exposure to light (lane L). The reactions were performed and analyzed as described in Figure 3 and Experimental Procedures. SR(1−4/5−7) was purified from transfected COS cells. R indicates location of the wild-type rhodopsin from photoreceptor outer segments; SR indicates location of the split rhodopsin mutant SR(1−4/5−7).

to light for 30 s and then mixed with rhodopsin kinase, [γ-^{32}P]ATP, and wild-type rhodopsin that had not been exposed to light. Only the light-activated SR(1−4/5−7) was phosphorylated when the incubation was carried out in the dark (Figure 3e). When the incubation was conducted under continuous illumination, wild-type rhodopsin became phosphorylated (lane L). Again, only the light-activated forms of rhodopsin were phosphorylated in this assay, and we found no evidence for trans-phosphorylation of dark rhodopsin.

To control for the possibility that native rhodopsin and/or native phospholipids are required for the reaction, we substituted urea-stripped bovine photoreceptor outer segments for wild-type COS cell rhodopsin in the trans-phosphorylation assay. As is shown in Figure 4, only light-activated rhodopsin was phosphorylated in the assay irrespective of whether native rhodopsin (Figure 4a) or SR(1−4/5−7) (Figure 4b) was selected for exposure to light. As before, both species were phosphorylated if the reaction was carried out under conditions of continuous illumination (lanes L).

We have shown previously that opsin mutants that constitutively activate transducin are also constitutively phosphorylated by rhodopsin kinase (Rim & Oprian, 1995). One of these mutants, K296G, in which the active site Lys296 residue is change to Gly, was used in this study to determine if the constitutively active opsin could induce rhodopsin kinase to phosphorylate SR(1−4/5−7) in the dark. This variation of the trans-phosphorylation reaction required no prior exposure of the proteins to light. Vesicles containing K296G opsin were simply mixed with rhodopsin kinase, [γ-^{32}P]ATP, and vesicles containing SR(1−4/5−7), and the incubation allowed to proceed for 40 min in the dark. As is shown in Figure 5, only the constitutively active K296G

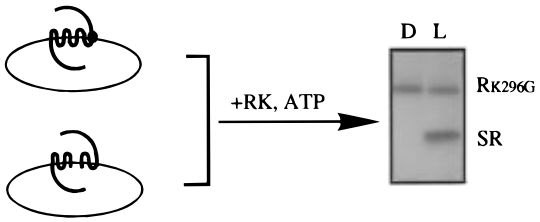

FIGURE 5: Assay for trans-phosphorylation of SR(1−4/5−7) using the constitutively active rhodopsin mutant K296G to activate rhodopsin kinase in the dark. Vesicles containing the full-length, constitutively active opsin mutant K296G (site of mutation in the seventh transmembrane segment is indicated by a solid circle) were mixed and incubated with rhodopsin kinase, [γ-^{32}P]ATP, and vesicles containing the split rhodopsin mutant SR(1−4/5−7) either in the dark (lane D) or under continuous exposure to light (lane L). K296G and SR(1−4/5−7) were purified from transfected COS cells and separately reconstituted into asolectin vesicles before assaying for phosphorylation by rhodopsin kinase. Conditions were as described in Figure 3 and Experimental Procedures. R_{K296G} indicates location of the K296G mutant; SR indicates location of the split rhodopsin mutant SR(1−4/5−7).

opsin was phosphorylated by rhodopsin kinase in the dark (lane D). Both proteins, K296G and SR(1−4/5−7), were phosphorylated if the reaction was performed under conditions of continuous exposure to light (lane L).

Up to this point the trans-phosphorylation assays were performed with light-activated and dark rhodopsin samples contained in different vesicles. Therefore, we have tested models for high-gain phosphorylation that require dissociation and diffusion of the activated kinase from a rhodopsin/rhodopsin kinase complex so as to encounter a second molecule of rhodopsin which has not been activated by light. It is possible, however, that the kinase does not dissociate from light-activated rhodopsin but rather diffuses as a complex with rhodopsin within the rod disk membrane until it encounters a second rhodopsin molecule which then becomes phosphorylated even if it has not itself been activated by light. A test of this model necessitates that activated rhodopsin and dark rhodopsin be present in the same vesicle, an experimental arrangement that is impossible to control using light-activated forms of the protein but which is made trivial by the availability of the constitutively active mutant opsin K296G.

When K296G and SR(1−4/5−7) were co-expressed, purified, and reconstituted into the same asolectin vesicles and then incubated with rhodopsin kinase and [γ-^{32}P]ATP in the dark, only K296G was phosphorylated (Figure 6a, lane D), whereas both K296G and SR(1−4/5−7) were phosphorylated when the incubation was carried out under continuous illumination (lane L). In this experiment, the concentrations of K296G and SR(1−4/5−7) were 10-fold greater than the estimated concentration of asolectin vesicles (see Experimental Procedures), making it highly likely that at least some of the vesicles contained both proteins. To increase this likelihood further, the vesicles were subjected to a freeze−thaw cycle to induce membrane fusion, and then assayed for trans-phosphorylation. As shown in Figure 6b, the freeze−thaw cycle was without apparent affect on the reaction. K296G, but not SR(1−4/5−7), was phosphorylated in the dark (lane D), whereas both K296G and SR(1−4/5−7) were phosphorylated when the reaction was carried out under continuous illumination (lane L). Therefore, we were unable to demonstrate trans-phosphorylation of rhodopsin even among molecules contained within the same vesicle.

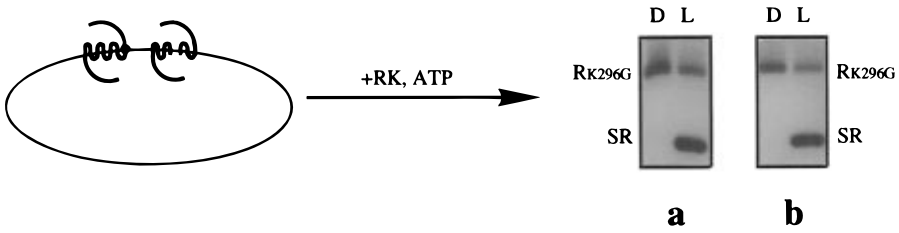

FIGURE 6: Assay for trans-phosphorylation of SR(1−4/5−7) using the constitutively active rhodopsin mutant K296G in the same vesicle to activate rhodopsin kinase in the dark. (a) The split rhodopsin mutant SR(1−4/5−7) and the constitutively active mutant K296G were co-expressed in COS cells, purified using immunoaffinity chromatography, and then reconstituted into asolectin vesicles before mixing with rhodopsin kinase and [γ-^{32}P]ATP and assaying for phosphorylation in the dark (lane D) or after exposure of the sample to light (lane L). (b) Same as in a except that the asolectin vesicles were subjected to a freeze−thaw cycle to induce fusion before mixing with rhodopsin kinase and ATP. Conditions were as described in Figure 5 and in Experimental Procedures. R_{K296G} indicates location of the K296G opsin mutant; SR indicates location of the split rhodopsin mutant SR(1−4/5−7).

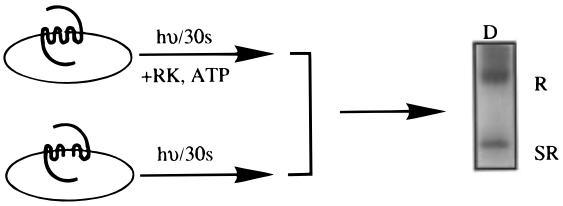

FIGURE 7: Assay to detect sequestration of rhodopsin kinase by light-activated rhodopsin. Wild-type rhodopsin in asolectin vesicles was mixed with rhodopsin kinase and [γ-^{32}P]ATP, exposed to light for 30 s, and then mixed and incubated in the dark (lane D) with vesicles containing SR(1−4/5−7) that had also been exposed to light for 30 sec, but not in the presence of rhodopsin kinase and [γ-^{32}P]ATP. Both wild-type and mutant rhodopsin were purified from transfected COS cells. Conditions were as described in Figure 3 and Experimental Procedures. R indicates location of the wild-type rhodopsin; SR indicates location of split rhodopsin mutant SR-(1−4/5−7).

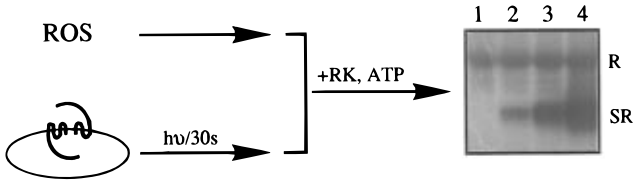

FIGURE 8: Assay for trans-phosphorylation of rhodopsin using high concentrations of rhodopsin and rhodopsin kinase. Wild-type rhodopsin in bovine photoreceptor outer segments was mixed with rhodopsin kinase, [γ-^{32}P]ATP, and vesicles containing SR(1−4/5−7) that had been exposed to light for 30 s, and the mixture was then incubated for 40 min in the dark. Reactions were performed and analyzed as described in Figure 3 and Experimental Procedures. Wild-type rhodopsin from photoreceptor outer segments was present at 25 μM final concentration and rhodopsin kinase at 50 nM. Final concentrations of SR(1−4/5−7) were as follows: lane 1, none; lane 2, 25 nM; lane 3, 100 nM; and lane 4, 500 nM. R indicates location of the wild-type rhodopsin from photoreceptor outer segments; SR indicates location of split rhodopsin mutant SR(1−4/5−7).

One possible explanation for our inability to detect trans-phosphorylation in these assays is that light-activated rhodopsin and rhodopsin kinase formed a complex that was so stable that dissociation did not take place on the time scale of these experiments and the kinase was effectively sequestered from interactions with other molecules (recall that rhodopsin was present in 50-fold molar excess over the kinase). To test for this possibility, vesicles containing wild-type rhodopsin were combined with rhodopsin kinase and [γ-^{32}P]ATP and exposed to light to activate rhodopsin and allow formation of a complex between the two proteins. The mixture was then mixed and incubated in the dark with SR(1−4/5−7) that had also been photoactivated by exposure to light for 30 s, but in the absence of rhodopsin kinase and [γ-^{32}P]ATP. As is shown in Figure 7, both the wild-type rhodopsin and SR(1−4/5−7) were phosphorylated under these conditions demonstrating clearly that rhodopsin kinase was not sequestered in the assay.

Previous studies of the high-gain phosphorylation reaction [e.g., Chen et al. (1995) and Dean and Akhtar (1996)] have used much higher concentrations of rhodopsin (typically 25 μM) and rhodopsin kinase (typically 50 nM) than the concentrations routinely used here (500 nm and 1 nM, respectively). As is detailed in Experimental Procedures and in Figure 1, the higher concentrations produce a significant dark reaction that can interfere with an analysis of trans-phosphorylation, and, for this reason, we elected to perform routine assays at lower concentrations of rhodopsin and rhodopsin kinase (500 and 1 nM, respectively). However, because of the clear possibility that our inability to observe trans-phosphorylation was a consequence of using the lower concentrations, we tested the higher concentrations for effect on the assay. When 25 μM rhodopsin in urea-stripped bovine photoreceptor outer segments was incubated with 50 nM rhodopsin kinase and [γ-^{32}P]ATP, a small but clearly observable amount of rhodopsin was phosphorylated in the dark (Figure 8, lane 1; we estimate that this corresponds to about 0.1% of the total rhodopsin on the basis of a comparison with the signal in lane 2). When increasing amounts of light-activated SR(1−4/5−7) (25, 100, and 500 nM, corresponding to the equivalent of a 0.1%, 0.4%, and 2.0% bleach, respectively) were added to the reaction, both the native rhodopsin and SR(1−4/5−7) were observed to be phosphorylated. However, phosphorylation of native rhodopsin is not significantly increased over that observed in the absence of SR(1−4/5−7). These results show that little, if any, trans-phosphorylation had taken place despite the higher rhodopsin kinase and rhodopsin concentrations.

DISCUSSION

The goal of this study was to develop an *in vitro* assay to directly test the model for trans-phosphorylation of rhodopsin by rhodopsin kinase. The key to the assay is the ability to biochemically distinguish molecules of rhodopsin that have been activated by light from those that have not been activated. For this purpose we chose a split rhodopsin mutant, SR(1−4/5-7), in which the protein is assembled in functional form from two separately expressed fragments (Yu et al., 1995). This mutant protein displays essentially wild-type behavior in spectral properties of the pigment, light-dependent activation of transducin, and light-dependent

phosphorylation by rhodopsin kinase [Figure 2 and Yu et al. (1995)]. However, since the protein is split in the extracellular loop connecting transmembrane segments 4 and 5, and since all of phosphorylation sites are located on the small carboxy-terminal fragment (composed of amino acid residues 197−348), SR(1−4/5−7) is easily distinguished from wild-type rhodopsin by electrophoretic mobility on SDS−PAGE gels. Thus, phosphorylation of SR(1−4/5−7) and wild-type rhodopsin can be monitored simultaneously in the same reaction mixture. An advantage of this system is that the experimental protocol may be executed using either the wild-type or the SR(1−4/5−7) mutant as the light-activated form of rhodopsin. Another advantage is that the experiments can be done using recombinant rhodopsin that is free of contaminating cone opsins.

A clear disadvantage of this system is that the two proteins must be physically separated during the light activation step. This precludes monitoring of the trans-phosphorylation reaction between molecules located within the same lipid vesicle. For these reactions we employed a second rhodopsin mutant, K296G, in which the apoprotein or opsin form is constitutively activated and, as a consequence, phosphorylated by rhodopsin kinase (Robinson et al., 1992; Rim & Oprian, 1995). Thus, the use of K296G allows trans-phosphorylation of SR(1−4/5−7) to be followed in darkness when SR(1−4/5−7) and K296G are reconstituted into the same lipid vesicles.

Despite a great effort and the use of several different experimental configurations, we have been unable to demonstrate trans-phosphorylation of dark rhodopsin by rhodopsin kinase in this system. It is not clear at this time why our results appear to differ from those reported earlier for high-gain phosphorylation reactions (Binder et al., 1990; Dean & Akhtar, 1996), including *in vitro* studies using urea-stripped photoreceptor outer segments and purified rhodopsin kinase (Chen et al., 1995). It is possible that contaminating cone outer segments are responsible for the high-gain phosphorylation. Alternatively, it is possible that additional components, present in rod photoreceptor outer segments but absent from our system, are required (Binder et al., 1990). If this is the case, the trans-phosphorylation assay may be useful for following purification of these additional components.

Our results also appear to be at odds with the experiments of Fowles et al. (1988) and Palczewski et al. (1991) in which a synthetic peptide from rhodopsin was shown to be efficiently phosphorylated by rhodopsin kinase only when light-activated rhodopsin was also present in the reaction mixture. Our data in Figure 8 show that the ability of dark rhodopsin to be phosphorylated by rhodopsin kinase in the assay was not significantly improved by the presence of light-activated SR(1−4/5−7). It is not clear at this time why the experiments with dark rhodopsin appear to differ from those with the synthetic peptide, but future work will address this issue.

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